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Investigation of Fungi Causing Twig Blight Diseases on Peach Trees in South Carolina

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INVESTIGATION OF FUNGI CAUSING TWIG BLIGHT DISEASES ON PEACH TREES IN SOUTH CAROLINA

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ABSTRACT

In 2016, fungal pathogens causing twig blight disease were isolated from symptomatic one-year-old shoots from peach orchards in 6 locations in South Carolina. Four twig blight pathogens, which included *Phomopsis amygdali*, *Botryosphaeria obtusa*, *Leucostoma persoonii,* and *Cytospora* sp., were isolated. *L. persoonii* was isolated in the highest frequency, followed by *P. amygdali* and *B. obtusa*. All pathogens were sensitive to thiophanate-methyl (FRAC 1), pyraclostrobin, and azoxystrobin (both FRAC 11). However, they were not sensitive to boscalid and fluopyram (both FRAC 7). *L. persoonii* exhibited less sensitivity to difenoconazole and propiconazole (both FRAC 3) while *P. amygdali* and *B. obtusa* were sensitive. *L. persoonii* was most virulent of the species based on the average necrotic area of fungal growth on detached, two-year-old wood of 4 peach cultivars exhibiting varying disease resistance. 'O'Henry' [bacterial spot (BS)-susceptible] was the most susceptible to *B. obtusa* when compared to 'Summerprince' (BS-resistant), 'Coronet' [brown rot (BR)-susceptible], and 'Contender' (BR-resistant) but was the least susceptible to *L. persoonii*. Additionally, 'Coronet' was the most susceptible to *L. persoonii*. There were no significant differences between cultivar susceptibility to *P. amygdali*.

In 2017, *L. persoonii* isolates were collected from scaffold limbs from 5 locations. High genetic variability of ITS1-5.8S-ITS2 was observed in *L. persoonii* isolates from both the 2016 and 2017 collections. The isolates were classified into genotypes (G) 1 to 6 based on how they clustered in a phylogenetic tree. Three of the genotypes (G2, G3, and G6) were isolated in the highest frequency in both years. Several morphology types were

also observed between and within genotype. All isolates were sensitive to thiophanate-methyl (FRAC 1) but were not sensitive to fluopyram and boscalid (FRAC 7). No significant differences in EC_{50} were observed between genotypes.

This research indicates the presence of three main twig blight pathogens in peach orchards in South Carolina with the genetically diverse *L. persoonii* being currently most prolific. Fungicide assay information indicates that some fungicidal active ingredients were effective in inhibiting mycelial growth of all twig blight pathogens. These data also provide information about cultivar tolerance in that resistance to twig blight pathogens is not necessarily connected to resistance to other peach pathogens.

DEDICATION

First and foremost, I dedicate this work to the Lord. I am thankful that this project allowed me to explore even the most microscopic beauty of His creation. I would also like to dedicate this work to my husband, Daniel, who loves me unconditionally and constantly supports and encourages me. I also dedicate this work to my parents, Keith and Jennie Hudgins, and brother, Thomas; I would not be who I am today without your love, encouragement, and guidance.

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TABLE OF CONTENTS

Page

Table of Contents (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

Table Page

CHAPTER 1

LITERATURE REVIEW

Diseases of Peach

Stone fruits (*Prunus* sp.) are economically important crops worldwide that include peach, nectarine, apricot, plum, and both sweet and sour cherry (Ogawa et al. 1995). Of all the stone fruits, peaches and nectarines are grown the most worldwide with China, Italy, and Spain being the largest producers in 2016 (FAOSTAT 2018). Because of their worldwide importance, it is imperative to understand diseases that can impact both yield and tree health. There is a litany of pathogens – fungi, bacteria, viruses, oomycetes, or nematodes – that infect various parts of peach trees. Leaf, flower, fruit, root, and wood diseases are all issues on peach trees at varying degrees. Additionally, many of these diseases are common to other stone fruits. Certain diseases cause issues preharvest while others affect the postharvest quality of peach fruit. Some pathogens can even cause disease symptoms and signs on multiple parts of a tree. Brown rot of peach is arguably the most well-known disease that mainly causes rot of fruit but may also cause blossom or twig blight (Adaskaveg, Schnabel, and Foerster 2008; Ogawa et al. 1995). In many production areas, peach scab and bacterial spot can cause fruit, leaf, and wood damage that threaten peach yields (Adaskaveg et al. 2008; Ogawa et al. 1995). Armillaria root and crown rot can causes major damage to peach orchards in the southeastern United States (Savage et al. 1953; Schnabel et al. 2005). Other diseases like bacterial canker, fungal gummosis, constriction canker, and Leucostoma canker are all wood diseases that cause dieback of shoots, limbs, and entire trees (Ogawa et al. 1995). While diseases like

brown rot and peach scab can be curbed by chemical control, it is much more difficult to control wood diseases in this way. Cultivar selection is recommended for preventing twig blight infections, but very little concrete research exists that suggests which cultivars are disease resistant. Literature suggests that cultural controls are the main management practices for these wood diseases (Adaskaveg et al. 2008). This requires a much more preventative focus on tree health and stress reduction. The purpose of this review is to explore the fungal pathogens causing twig blight diseases of peach.

Twig Blight of Peach

Symptoms and Signs. Causes of twig blight can be biotic and abiotic. Abiotic causes of twig blight include nutrient deficiencies, drought stress, and lack of sunlight especially due to canopy shade (Adaskaveg et al. 2008; Proebsting and Middleton 1980). Generally, though, twig blight is associated with some sort of biotic disease. This could include bacterial canker (Cameron 1962), which is caused by *Pseudomonas syringae* van Hall, or a multitude of fungal diseases. Fungal twig blight symptoms and signs are often pathogen-specific. However, these pathogens share common symptoms and signs such as canker formation on peach shoots or trunks, shoot or limb dieback, and potentially death of entire trees (Beckman et al. 2003; Lalancette and Robison 2002; Luepschen et al. 1979; Tekauz and Patrick 1974). The main sign of the disease is the formation of asexual fruiting bodies called pycnidia in spring and during summer, which form on the deadened tissue of the tree and produce cirri bearing conidia under appropriate conditions (Bertrand 1976; Haenseler and Daines 1941; Lalancette and Robison 2001; Weaver 1974). While

symptoms like cankers and dieback cannot distinguish between fungal twig blight pathogens, pycnidia, cirri, and conidia can be diagnostic upon laboratory examination.

Economic Importance. Over time, twig blight on peach can become a serious problem. After several growing seasons with twig blight infections on peach trees, trees grow slower and fruit yield is reduced (Beckman, Pusey, and Bertrand 2003). Fungal gummosis has long been considered an issue in the southern United States (Britton and Hendrix 1982; Weaver 1974). As for its impact on yield, one study indicated that peach yield of trees with fungal gummosis was 11.5 to 22.5% lower with product value 14.7 to 19.4% lower than a healthy orchard (Ezra, Hershcovich, and Shtienberg 2017). Constriction canker has been found mainly on the east coast of the United States, both in the south (Farr, Castlebury, and Pardo-Schultheiss 1999; Uddin, Stevenson, and Pardo-Schultheiss 1997) and north (Haenseler and Daines 1941). A study examining the economic impact of constriction canker on peach revealed a 21 to 28% yield loss, indicating the need for control of the pathogen in severely affected orchards (Lalancette and Polk 2000). In the case of Leucostoma canker, northern and western states such as New York, Illinois, Colorado, and Idaho historically have experienced large losses due to the disease (Gairola and Powell 1970). Additionally, southern states have also dealt with Leucostoma canker (Adams, Surve-Iyer, and Iezzoni 2002; Alfieri, Seymour, and French 1974; Hammar 1989), but the disease has not yet been recognized as a major issue. In general, because twig blight pathogens can cause the loss of entire trees, and consequently a loss of yield, these diseases are important to prevent and manage.

Fungal Causal Agents of Twig Blight on Peach

Botryosphaeria obtusa and B. dothidea. The two causal agents of fungal gummosis of peach are *Botryosphaeria dothidea* (Moug. Ex Fr.) Ces. & De Not. and *Botryosphaeria obtusa* (Schwein.) Shoemaker (Britton and Hendrix 1982; Weaver 1974). *B. dothidea* was first observed on peach by Stevens in 1926 but the fungus was not associated with a disease at this time (Stevens 1926). Additionally, it was considered *B. ribis* until a name clarification in 1963 (Witcher and Clayton 1963). Later, *B. dothidea* was eventually associated with the gummosis disease of peach trees in 1974 in Georgia (Weaver 1974). *B. dothidea* was long considered the main causal agent of fungal gummosis until 1982 when Britton and Hendrix determined that there were three causal agents of gummosis – *B. dothidea, B. obtusa*, and *B. rhodina* (Britton and Hendrix 1982). The most common species of *Botryosphaeria* in the southeastern United States is *B. obtusa* (Ogawa et al. 1995).

Conidia of *B. obtusa*, on average, are 8 to 13 x 19 to 27 μm (Choueiri et al. 2006; Chattaoui et al. 2012; Kaliterna et al. 2011; Úrbez-Torres et al. 2006; Yan et al. 2011). *B. obtusa* conidia are ovoid with either rounded or truncated bases, aseptate, and appear hyaline when first developed but turn dark brown during maturation (Kaliterna et al. 2011; Yan et al. 2011; Chattaoui et al. 2012). Sexual fruiting bodies of *B. obtusa* – and all species in the *Botryosphaeriaceae* – are perithecia (Mehl et al. 2013). Perithecia of Botryosphaeriaceae contain asci that bear eight ascospores (Weaver 1974).

B. dothidea produces pycnidia (asexual fruiting structures) on diseased plant tissue that exude conidia under wet, warm conditions (Weaver 1979). These conidia, on

average, are 4.5 to 8 x 15 to 26 μm (Weaver 1974; Valencia-Botín et al. 2003; Jurc et al. 2006). Typical conidia are hyaline, ellipsoidal or fusiform, and aseptate (Valencia-Botín et al. 2003; Weaver 1974). Perithecia of *B. dothidea* surround clavate asci bearing eight ascospores (Weaver 1974). These ascospores may be ovoid, fusoid, or ellipsoid and are typically hyaline and aseptate but may become darker and develop septation with maturity (Phillips et al. 2013).

The disease cycle of these *Botryosphaeria* spp., like other twig blight pathogens, begins with infection of the wooden tissue through a wound or opening in the wooden tissue. Conidia of *Botryosphaeria* spp. can infect through lenticels in wooden tissue (Weaver 1974). Additionally, conidia may also enter the wooden tissue through pruning or other mechanical wounds (Pusey 1989). Research has also suggested that *Botryosphaeria* spp. of peach, with *B. obtusa* being isolated in the highest frequency, are also able to infect through buds, but that this is not the main infection pathway for the gummosis disease (Britton and Hendrix 1989). Once the pathogen is established in the wood, it will produce sunken lesions near the infection point (typically lenticels), brown necrotic lesions under the bark, blister-like protrusions on the bark surface containing necrotic tissue, and exudation of gum from lenticels (Weaver 1974). This gumming is often triggered by a rain event and usually occurs in severe infections (Beckman, Pusey, and Bertrand 2003). As the disease matures, pycnidia – and sometimes perithecia – are formed inside lenticels (Weaver 1974). Overtime, trees with fungal gummosis experience dieback of twigs, shoots, and limbs that weakens trees and eventually kills them (Beckman et al. 2003; Ezraet al. 2017).

Phomopsis amygdali. The main causal agent of constriction canker of peach is *Phomopsis amygdali* (Delacr.) J.J. Tuset & M.T. Portilla (Tuset and Portilla 1989; Farr et al. 1999). This pathogen was originally described as *Fusicoccum amygdali* Delacr. in 1905 and was first observed on peach in 1953 (Guba 1953). It was reclassified as *P. amygdali* in 1989 (Tuset and Portilla 1989). The pathogen can also cause a fruit rot, but this is less common than twig blight (Cohoon and Daines 1954).

P. amygdali produces two types of conidia – α-conidia and β-conidia (Tuset and Portilla 1989). However, β-conidia of *P. amygdali* are very rarely produced in culture and are typically only found in the field under certain conditions (Tuset and Portilla 1989). The α-conidia are hyaline, non-septate, straight, and fusiform while β-conidia are hyaline, usually straight or slightly curved, and filiform (Tuset and Portilla 1989). On average, α-conidia range anywhere within 5 to 10 x 1.5 to 4 μm and β-conidia 13 to 30 x 1 to 2 μm (Dai et al. 2012; Tuset and Portilla 1989; Uddin et al. 1997).

P. amygdali infections can occur in spring through bud scales, stipules, and fruit scars as well as blossoms or in fall through leaf abscission scars (Cohoon and Daines 1956). In the fall, infections are more likely to occur if the tree experiences an early defoliation (Cohoon and Daines 1956). Additionally, late spring infections and early fall infection (i.e. infections occurring soon after leaf fall) typically result in more severe infections (Cohoon and Daines 1956). In theory, infection can occur year-round because conidia are produced all year (Lalancette and Robison 2001). Early descriptions have suggested that any wounding or opening in the wooden tissue will allow for infection on all peach cultivars (Cohoon and Daines 1956). Upon entrance of conidia into a wood

opening, a small canker forms around the node (Haenseler and Daines 1941). These cankers appear water-soaked with undefined edges at first but become reddish-brown and sunken with more defined edges as they mature (Haenseler and Daines 1941; Lalancette and Robison 2002). As cankers mature even further, they become hard and gray and eventually produce black pycnidia (Haenseler and Daines 1941). By the summer after infection, the vascular tissue of the infected shoots is entirely restricted, cutting off supply to water and nutrients and resulting in shoot death (Lalancette and Robison 2002). These shoots are typically fruit-bearing, meaning that if the shoots are lost, then the tree loses yield (Lalancette and Robison 2002; Uddin and Stevenson 1998). The pycnidia that form on cankers eventually sporulate and produce cream-colored cirri bearing conidia that provide inoculum to infect new shoots, which prolongs the disease cycle and leads to infections on a larger scale (Haenseler and Daines 1941; Lalancette and Robison 2001).

Leucostoma persoonii* and *L. cintca. Leucostoma canker of stone fruits, also commonly referred to as cytospora canker or perennial canker, is caused by *Leucostoma persoonii* (Nitschke) Höhn [synonym: *Cytospora leucostoma* (Pers.) Sacc.] and *Leucostoma cinctum* (Fr. : Fr.) Höhn. (synonym: *Cytospora cincta* Sacc.). These pathogens causing this canker disease were originally described as *Valsa leucostoma* (Pers.) Fr. and *Valsa cincta* until their reclassification in 1928 (Willison 1936).

The conidia produced by these species measure 5 to 10 x 1 to 2 μm and are hyaline and allantoid (Ogawa et al. 1995; Norphanphoun et al. 2017; Willison 1936). The sexual fruiting bodies of these species are perithecia containing asci bearing eight

ascospores (Ogawa et al. 1995). The ascospores are also hyaline and allantoid and measure 15 to 30 x 2 to 5 μm (Ogawa et al. 1995; Willison 1936).

Leucostoma spp. are known in particular for being opportunistic on weakened hosts (Hildebrand 1947) (e.g. a tree infected with another twig blight pathogen or a tree experiencing drought). Interestingly, early research on these fungi indicated that *L. persoonii* is less likely to be a primary pathogen than is *L. cinctum*, but this is not always the case (Willison 1936). Like other fungal twig blight pathogens, *Leucostoma* spp. require a wound in the wooden tissue through which to enter to cause infection (Luepschen et al. 1979; Tekauz and Patrick 1974). The most common infection site is a pruning wound (Tekauz and Patrick 1974; Willison 1933). Winter injuries as well as leaf scars at the nodes are also a common infection court for the pathogens (Tekauz and Patrick 1974). Infection can occur anywhere on the tree – high up in smaller shoots or lower on the tree in large limbs and the trunk (Luepschen et al. 1979). Upon infection, a sunken, brown canker forms around the infected area (Willison 1933). After establishment, gumming appears in and around the canker (Hildebrand 1947). Mature cankers are black and desiccated with wood beginning to separate from inner and surrounding wood (Willison 1933). The tree may also produce callus around the cankers in growing seasons thereafter which causes rings around the original canker (Hildebrand 1947; Willison 1933). As the disease progresses, the vascular tissues are interrupted which causes shoot dieback (Tekauz and Patrick 1974). Pycnidia are produced on and around the canker which will sporulate orange cirri under humid and warm conditions, providing inoculum to infect other shoots or trees (Hildebrand 1947).

Management Strategies

Cultural. For all twig blight pathogens, the most common strategy for managing the disease is to prune out and destroy infected shoots (Alfieri et al. 1974; Beckman et al. 2003; Uddin and Stevenson 1998). In the southeastern United States, pruned twigs and branches are raked into the row middles, chopped into small and fast-degradable chunks using a flail mower, and left in the field for degradation and resupply of soils with nutrients. Pruning out and destroying infected shoots removes inoculum from the orchard, which helps prevent the spread of the disease. However, avoiding mechanical damage, specifically from pruning, is imperative for blocking fungal entrance into the wooden tissue in the first place (Alfieri et al. 1974). Therefore, attention must be paid to optimal pruning timing and technique. Pruning should occur right before blossoming, so late winter pruning is preferred to prevent infection (Alfieri et al. 1974). Studies have shown that pruning too close to the branch or leaving too long of stubs can increase infection and lead to more dieback (Biggs 1992). Additionally, it has been suggested that removal of unnecessary plant debris and maintaining overall orchard sanitation may also be effective in preventing inoculum from spreading (Uddin and Stevenson 1998). Sanitizing all tools, particularly pruning tools, is also important for preventing the spread of a twig blight disease from one tree to another (Alfieri et al. 1974). Because twig blight can be exacerbated by abiotic stressors, irrigation and nitrogen management of trees should be priorities (Alfieri et al. 1974; Simoes et al. 2010). Cultural control is the main recommended management practice for twig blight diseases (Blaauw et al. 2018; Adaskaveg et al. 2008).

Cultivar Selection. Progress is being made in understanding cultivar tolerance to wood pathogens, including twig blight. One study on tolerance of cultivars to fungal gummosis, caused by *B. dothidia*, determined that disease tolerant cultivars typically produce poor quality fruit and lower yield or exhibit some other trait qualities that make them undesirable to grow (Okie and Pusey 1996). However, these cultivars can be used as a source of tolerance in breeding favorable disease tolerance traits into commercial cultivars. Another study suggested that favorable disease tolerance traits may already exist in commercial cultivars (Beckman and Reilly 2005). For example, 'Redskin' was found to be more tolerant than 'Summergold' (Beckman and Reilly 2005). This same study also revealed that the most susceptible cultivars made up 35% of the peach production in the southeast at that time (Beckman and Reilly 2005). However, recently, a key locus from almond associated with tolerance to *B. dothidea* was described that may help with marker-assisted selection and introgression of this resistance trait into peach (Mancero-Castillo et al. 2018).

Research on cultivar tolerance to *P. amygdali*, the causal agent of constriction canker, is rare. Based on the one source identified in the literature, 'J.H. Hale', 'Coronet', and 'Dixired' are considered less susceptible to constriction canker compared to 'Redgold', 'Redhaven', and 'Golden Jubilee' (Ogawa et al. 1995). Curiously, many extension publications suggest selecting tolerant cultivars, but almost none explicitly say which cultivars are tolerant.

Cultivars such as 'Ozark,' 'Envoy,' 'Comanche,' 'Elberta,' 'Redhaven,' and 'Redglobe' have shown medium-to-high levels of tolerance to Leucostoma canker,

caused primarily by *L. persoonii* (Gairola and Powell 1970). The most promising connection to Leucostoma canker tolerance has been found in cold-tolerant peach cultivars; one study suggested that the cold-tolerant cultivar Yennoh is tolerant to *L. persoonii* and *L. cinctum* (Iezzoni et al. 1992). Additionally, the study suggested that this cultivar could be crossed with a Leucostoma-susceptible cultivar and produce similar tolerance, indicating that the trait is heritable (Iezzoni et al. 1992). However, cold-tolerance is mainly important for northern climates, not the peach-producing southeastern United States.

Another interesting sign of twig blight disease tolerance is the production of suberin by the tree. Many studies have determined that the wounding of plant tissue causes the formation of a lignosuberized layer, which is an important component of preventing fungal infection (Biggs and Britton 1988; Biggs and Miles 1985, 1988; Biggs and Peterson 1990; Rittinger et al. 1987). One study in particular indicated that cultivars that produced suberin sooner after wood damage were typically more tolerant to infection by *Leucostoma* spp. (Biggs and Miles 1985; Biggs and Peterson 1990). This feature of certain cultivars may help in future research or breeding of cultivars for twig blight tolerance because suberin deposition as a plant response aids in the healing of wounds to the wooden tissue (Biggs and Miles 1985).

Chemical. Because of the disease cycle and nature of these pathogens, chemical control is mainly preventative. Much of the available information pertains to fungicides to which pathogens have developed resistance over time or that are no longer registered for use. The 2018 Southeastern Peach, Nectarine, and Plum Pest Management and

Culture Guide does not list any chemical control options for twig blight pathogens, and did not list *L. persoonii/L. cinctum* as a pathogens affecting peach in the southeast (Blaauw et al. 2018).

While still not vastly studied, recent research on chemical control of *Botryosphaeria* spp. and *P. amygdali* suggested captafol to be more effective than captan in decreasing incidence of fungal gummosis (Beckman et al. 2003). Another study showed that, of the fungicides tested, propiconazole was most effective in controlling *B. obtusa* and that azoxystrobin was not effective (Ji et al. 2012). One study indicated that fungicides such as carbendazim (FRAC 1), prochloraz, and difenoconazole (both FRAC 3) were effective in managing *P. amygdali* in a laboratory setting (Ji et al. 2013). Additionally, captan (FRAC M04), chlorothalonil (FRAC M05), and azoxystrobin (FRAC 11) were once shown to provide a level of control of *P. amygdali* in the field but no fungicide was able to provide more than 75% control (Lalancette and Robison 2002).

The research on fungicides for *Leucostoma* spp. control is out of date. One study showed that benomyl (FRAC 1) and captafol (FRAC M04) were effective for controlling *L. persoonii* (Northover 1992), which disagreed with a previous study that found no benefit of using benomyl (Grosclaude 1985). Many plant pathogens have evolved to become resistant to benomyl or to any other methyl benzimidazole carbamates fungicides (Bernstein et al. 1995; Penrose and Koffman 1977), which may explain conflicting results mentioned above. Both, benomyl and captafol are no longer registered for use on peach. While much of this research is useful, there has not been an up-to-date, comprehensive

study of the efficacy of the most commonly used fungicides applied currently in peach production in the southeastern U.S. on twig blight fungi.

In summary, the basic biology of twig blight pathogens (e.g. morphology, infection pathways, disease cycles, etc.) appear to be well studied; however, there are knowledge gaps with regard to twig blight pathogen management. Pathologists have previously studied fungicides that may control twig blight pathogens, but many of these chemicals are no longer registered for use. Additionally, cultivars that have been tested in the past are not currently grown in the southeastern United States. Because of the lack of current research on twig blight pathogens and their management pertaining to the southeastern U.S., in addition to our own observations of peach orchards in South Carolina in recent years, it is important to continue to investigate these pathogens and explore management options.

Objectives of Study

The objectives of this study were to (i) identify the species of fungi causing twig blight on peach trees in South Carolina, (ii) determine their sensitivity to commonly-applied and modern fungicides, and (iii) assess susceptibility of peach cultivars sensitive or tolerant to other diseases of peach, including bacterial spot and brown rot, to twig blight fungi.

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CHAPTER 2

INVESTIGATION OF FUNGI CAUSING TWIG BLIGHT DISEASES ON PEACH TREES IN SOUTH CAROLINA

Abstract

A survey of fungal pathogens causing twig blight on commercial peach trees was conducted in South Carolina in the fall of 2016. Shoots with cankers, pycnidia, and dieback were collected from six locations around the state. Isolates obtained from these samples were identified as *Botryosphaeria obtusa*, *Phomopsis amygdali*, *Leucostoma persoonii*, and *Cytospora* sp., based on colony morphology, conidia size and shape, and ribosomal DNA sequence analysis. *L. persoonii* was the most prevalent species and was isolated in five of the six locations, followed by *P. amygdali* and *B. obtusa.* The sensitivity of representative isolates of *B. obtusa*, *P. amygdali*, and *L. persoonii* to fungicides of different FRAC codes was evaluated. All species tested were sensitive to thiophanate methyl (FRAC 1) and pyraclostrobin and azoxystrobin (both FRAC 11), while all species were resistant to boscalid and fluopyram (both FRAC 7). *P. amygdali* and *B. obtusa* were sensitive to difenoconazole and propiconazole (both FRAC 3), while *L. persoonii* was moderately resistant. *L. persoonii* was the most virulent species based on expansion of mycelium in the cambium layer of two-year-old, detached twig pieces. Bacterial spot (BS)-sensitive cultivar O'Henry was most susceptible to *B. obtusa* compared to BS-resistant 'Summerprince', brown rot (BR)-resistant 'Contender' and BR-sensitive 'Coronet' but was least susceptible to *L. persoonii.* 'Coronet' was most

susceptible to *L. persoonii*. There were no significant differences between susceptibility of the cultivars to *P. amygdali*. This study established that *L. persoonii* is currently the most frequent twig blight pathogen in South Carolina, perhaps due to its superior fitness. Some fungicides were effective in controlling all twig blight pathogens and may therefore be useful for chemical management strategies. Our study also provides first evidence that the genetic basis of resistance to BS and BR in peach trees is not necessarily linked to tolerance to wood pathogens.

Introduction

Twig dieback reduces fruiting wood and therefore can impact yield potential in commercial peach [*Prunus persica* (L.) Batsch] orchards. Abiotic factors such as shading by a dense canopy, drought, or nutrient imbalances can cause shoots to die back (Johnson 2008; Proebsting and Middleton 1980). There are also several biotic causes of twig blight, including that caused by *Pseudomonas syringae* van Hall (Cameron 1962). However, most often, biotic twig blight is attributed to fungal pathogens. These pathogens require an opening in the wooden tissue through which to enter (Cohoon and Daines 1956; Luepschen et al. 1979; Pusey 1989; Tekauz and Patrick 1974). Because the pathogens are often opportunistic on weakened hosts, they may infect trees that have already been infected with another twig blight pathogen (Hildebrand 1947). Other research also indicates that multiple infections of twig blight canker pathogens can occur at the same time (Bai et al. 2015). Other factors such as insect damage, drought, and freeze damage also increase the probability of a twig blight fungal infection (Bertrand et

al. 1976; Dhanvantari 1978; Willison 1933). Other research has suggested that excess nitrogen increases susceptibility to twig blight (Simoes et al. 2010).

No systematic survey of twig blight-causing pathogens has been conducted in the southeast, but *Phomopsis amygdali* (Delacr.) J.J. Tuset & M.T. Portilla, *Botryosphaeria dothidea* (Moug. Ex Fr.) Ces. & De Not., *Botryosphaeria obtusa* (Schwein.) Shoemaker, and *Leucostoma persoonii* (Nitschke) Höhn have been reported to cause peach twig dieback elsewhere (Adams et al. 2002; Britton and Hendrix 1982; Farr et al. 1999; Hammar 1989). *P. amygdali* and *L. persoonii* have been reported to infect through leaf, bud scale, stipule, fruit scars and blossoms (Cohoon and Daines 1956; Gairola and Powell 1970; Hildebrand 1947; Tekauz and Patrick 1974). *Botryosphaeria* spp. have been reported to enter into wooden tissue through lenticels (Weaver 1974). Most twig blight fungi may also infect trees through wounds from pruning or other mechanical damage (Cohoon and Daines 1956; Luepschen et al. 1979; Pusey 1989). After infection of a shoot, a canker forms, matures, and ultimately kills the entire shoot (Lalancette and Robison 2001). Pycnidia then form on the canker and dying shoot, which, under wet or humid conditions, will produce cirri bearing conidia (Bertrand 1976; Haenseler and Daines 1941; Lalancette and Robison 2001; Weaver 1974). The conidia are dispersed by water and can spread disease throughout an orchard (Amponsah et al. 2009; Lalancette and Robison 2001; Luepschen and Rohrbach 1969). Symptoms of these diseases include cankers or lesions, gumming of wounded areas, darkening and necrosis of wooden tissue, twig or scaffold limb dieback, and, most severely, death of tree (Alfieri et al. 1974; Haenseler and Daines 1941; Uddin and Stevenson 1998; Weaver 1974).

Because of the similarities in symptom and sign expression, these twig blight pathogens often must be cultured in a lab instead of diagnosed in the field. Additionally, due to the fact that multiple species within a genus can cause a twig blight disease, the ITS regions are often sequenced to determine species identity. The sensitivity of many wood pathogens to recently registered fungicides is unknown and so is the susceptibility to dieback disease of newer cultivars with known resistance traits to other important peach diseases, such as brown rot and bacterial spot. The objectives of this study were to (i) identify the species of fungi causing twig blight on peach trees in South Carolina, (ii) determine their sensitivity to commonly-applied and modern fungicides, and (iii) assess susceptibility of peach cultivars sensitive or tolerant to other diseases of peach, including bacterial spot and brown rot, to twig blight fungi.

Materials and Methods

Sample collection and pathogen isolation. One-year-old peach twigs displaying pycnidia were collected from six locations in South Carolina (Chesnee, Greer, McBee, Mountain Rest, and York) during 2016. To isolate the fungi, necrotic tissue underneath the periderm was removed and surface sterilized in 10% sodium hypochlorite for 1 minute and rinsed in sterilized water for 1 minute. The tissue was then placed onto potato dextrose agar (PDA; 35.1 g Difco™ PDA, 900 ml H_2O) and incubated in the dark at 22°C until fungal mycelia developed. Single-hyphal isolates were made of these cultures and stored on filter paper as described previously (Hu, Cox, et al. 2011).

DNA Extraction, PCR amplification, and sequencing of the Internal Transcribed Spacer regions 1 and 2. Fungal DNA was extracted according to a previous protocol (Chi et al. 2009) except that mycelia were grown over cellophane strips on PDA to ease mycelia removal. Internal transcribed spacer (ITS) region 1, ribosomal 5.8S subunit, and ITS 2 were amplified through polymerase chain reaction (PCR) using the primers ITS1-F and ITS4. The PCR program consisted of 5 minutes at 94°C; 35 cycles of 40 seconds at 94°C, 1 minute at 55°C, and 2 minutes at 72°C; and 10 minutes at 72°C in a Bio-Rad T100™ Thermal Cycler. PCR products were subsequently sent to Arizona State University DNA Lab for Sanger sequencing. The returned sequences were compared to the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (nBLAST) to determine species identities. Sequencing editing, assembly, alignment, and phylogenetic tree-creation were conducted using Geneious (version 11.0.4, Biomatters).

Fungicide sensitivity assay. To determine fungicide sensitivity, the half maximal effective concentration (EC_{50}) was determined using representative fungicides from four Fungicide Resistance Action Committee (FRAC) mode-of-action (MOA) codes. Fungicides were selected based on grower-use in peach orchards. The fungicides used included thiophanate-methyl (methyl benzimidazole carbamates [MBC], FRAC 1); propiconazole and difenoconazole (demethylation inhibitors [DMI], FRAC 3); boscalid and fluopyram (succinate dehydrogenase inhibitors [SDHI], FRAC 7); and pyraclostrobin and azoxystrobin (quinone outside inhibitors [QoI], FRAC 11). Malt extract agar (MEA; 10 g malt, 15 g agar, 1 L H_2O) was amended with the above mentioned fungicides of

FRAC 1, 3, and 11. Minimal media (MM; 10 g glucose, 1.5 g K_2HPO_4, 2 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 2 g yeast extract, 12.5 g agar, 1 L H_2O) was used for FRAC 7 (Hu, Luo, et al. 2011). Media for FRAC 11 were additionally amended with salicylhydoxamic acid (SHAM) to block the alternative oxidase pathway. A preliminary study investigated the sensitivity of all isolates to 0.1, 1, and 10 µg/ml active ingredient (ai). Three 4 mm in diameter mycelial plugs were taken from the periphery of one to two-week-old cultures and placed on each fungicide-amended and unamended control petri dish and incubated in the dark at 22°C. Their average diameters were recorded when the three colonies were nearly touching. Upon evaluation of these, a second assay was conducted using refined concentrations. The concentrations 0.1, 0.3, and 1.0 µg/ml active ingredient (ai) were used for testing thiophanate-methyl (FRAC 1) for all pathogens except *B. obtusa.* For *B. obtusa*, 0.03, 0.1, 0.3, 1.0, and 3.0 µg/ml ai were used. Boscalid and fluopyram (FRAC 7) were tested at 3, 10, 30, and 100 µg/ml ai and pyraclostrobin and azoxystrobin (FRAC 11) at 0.01, 0.03, 0.1, 0.3, 1.0, and 3.0 µg/ml ai. Data from the preliminary study of propiconazole and difenoconazole (FRAC 3) were sufficient to calculate EC_{50}, so no further testing was conducted. The procedure aforementioned was repeated on these new concentrations. These data were used to determine the fungicide concentrations at which 50% of fungal growth was inhibited.

Cultivar susceptibility assay. Four peach [*Prunus persica* (L.) Batsch] cultivars were selected based on their tolerances to two common peach diseases, brown rot (*Monilinia fructicola*) and bacterial spot (*Xanthomonas arboricola* pv. *pruni*). The cultivars chosen were ‘Contender’ [tolerant to brown rot (Pacheco et al. 2014)], ‘Coronet’

(susceptible to brown rot), ‘Summerprince’ (resistant to bacterial spot), and ‘O’Henry’ (susceptible to bacterial spot). Three trees were selected per cultivar, and 2-year-old wood was collected to produce 3 experimental replicates per tree (Appendix A). Peach wood was obtained from the Clemson University Musser Fruit Research Center in Seneca, South Carolina. One representative isolate each of *B. obtusa*, *P. amygdali*, and *L. persoonii* were chosen for inoculations based on EC_{50} data; the isolates with highest EC_{50} for each pathogen were selected.

Isolates were grown on PDA from permanent culture for 8 days in the dark at 22°C. The collected wood was cut into approximately 12 cm sections. Wood section ends were sealed with Kilz 2® Latex primer to avoid desiccation. Twigs were sterilized for 5 minutes in 10% sodium hypochlorite solution, washed in sterilized distilled water for 1 minute, and allowed to dry in a laminar flow hood. A 4mm in diameter core borer was used to remove discs from wood periderm. Plugs of mycelia were placed over the wounded areas and sealed with Parafilm. Two inoculations of the same pathogen were made per wood section and measurements were averaged (Appendix B). Controls were prepared using plugs of sterile PDA. The wood was then placed inside one opaque plastic storage container in two layers separated by aluminum foil and allowed to incubate at room temperature for 7 days. The average necrotic area was estimated by multiplying length by width, and the entire experiment was repeated.

Statistical analysis. We used JMP (SAS Institute, Inc., Cary, NC, USA) for all statistical modeling. Multivariate statistics included multiple linear regression and correlation metrics to understand the data structure and interactions. A two-way ANOVA

analysis was used to determine the significance of pathogen, cultivar, and their interactions to disease lesion area. Where the full model, pathogen, and the interaction of pathogen and cultivar effect were statistically significant ($\alpha = 0.05$), we compared means of each cultivar by pathogen as well as combined cultivars for each pathogen using the Tukey HSD post-hoc comparison.

Results

A total of 111 isolates were collected from six locations in 2016 and grouped based on culture morphology on PDA. Morphology 1 was characterized by white mycelia that turned grey-green with age and grew, on average, 68mm in 6 days; morphology 2 by dark grey-green mycelia that also formed aerial mycelia and grew, on average, the entire diameter of the petri dish (85mm) within 6 days; morphology 3 by light yellow-green mycelia with no aerial extensions that turned green-brown with age and grew, on average, 83mm in 6 days; and morphology 4 by light-green mycelia with no aerial extensions that turned dark green with age and grew, on average58mm in 6 days (Fig. 1.1). These distinct colonies were later identified based on spore morphology as well as ribosomal DNA sequence analysis as *P. amygdali, B. obtusa, L. persoonii,* and *Cytospora* sp. *B. obtusa* conidia measured 15 to 26.25 x 6.25 to 10 µm and were ellipsoidal, non-septate, and initially hyaline but turned brown after maturation. *P. amygdali* α-conidia measured 3.75 to 7.5 x 2.5 to 5 µm and were straight, hyaline, non-septate, and fusiform in shape. No β-conidia were observed. Conidia of *L. persoonii* were 2.5 to 6.25 x 1.25 to 2.5 µm and were hyaline, non-septate, allantoid, and slightly curved.

The identity of *Cytospora* sp. was based solely upon DNA sequencing information. All species were found in at least two different locations. The most commonly found species by location were *L. persoonii* (5 locations) and *B. obtusa* (3 locations). The most frequently isolated species were *L. persoonii* (57 isolates) and *P. amygdali* (37 isolates; Table 1.1).

Colonies of different morphologies differed in ribosomal DNA sequences. The ITS regions of representative isolates of each distinct cultural morphology from each location were sequenced and revealed 4 fungal species known to cause twig blight on stone fruits. Morphology 1 sequences were highest in sequence identity to *P. amygdali* (100% identity to ITS accession sequence number NR_119753 in NCBI); morphology 2 sequences were highest in sequence identity to *B. obtusa* (99.9% identity to HQ629955); morphology 3 sequences were highest in sequence identity to *L. persoonii* (99.8% identity to AF191180); and morphology 4 sequences were highest in sequence identity to *Cytospora donetzica* (98.4% identity to KY417729). The latter sequences varied by 8 nucleotides in the ITS1 and 2 regions and thus we refer to these isolates as *Cytospora* sp. Because of this species-uncertainty, the *Cytospora* sp. isolates were excluded from further experimentation.

Representatives of species exhibited differences in sensitivity to fungicides. The EC_{50} values for thiophanate-methyl (FRAC 1) ranged from 0.13 to 0.156, 0.002 to 0.064, and 0.128 to 0.175 µg/ml for *P. amygdali, B. obtusa,* and *L. persoonii*, respectively (Table 1.2). For difenoconazole, the EC_{50} values ranged from 0.049 to 0.289, 0.026 to 0.207, and 0.812 to 1.037 µg/ml for *P. amygdali, B. obtusa,* and *L. persoonii*,

respectively. For propiconazole, the values ranged from 1.031 to 1.189, 0.094 to 0.585, and 0.929 to 2.284 µg/ml for *P. amygdali, B. obtusa,* and *L. persoonii*, respectively (Table 1.3), indicating that *L. persoonii* exhibited moderate resistance to both FRAC 3 fungicides. Overall, difenoconazole had higher intrinsic activity compared to propiconazole. All pathogens were resistant to FRAC 7 fungicides based on EC_{50} values for boscalid and fluopyram equal or greater than 56.819 µg/ml. Most isolates were sensitive to FRAC 11 pyraclostrobin and azoxystrobin with EC_{50} values less than 0.01 µg/ml. Azoxystrobin had higher intrinsic activity compared to pyraclostrobin. The highest EC_{50} of 0.611 µg/ml was identified for a *B. obtusa* isolate. These data suggest that all species were sensitive to FRAC 1 and FRAC 11 fungicides, that sensitivity to FRAC 3 fungicides was species dependent, and that FRAC 7 fungicides are ineffective (Appendix C).

Infection symptoms of the twigs were similar for all pathogens. Bark tissue appeared slightly sunken, and brown, necrotic lesions formed underneath the bark. Gumming occurred out of wounds of many twigs. While infection symptoms were alike, we identified differences in virulence and cultivar response between the three pathogens investigated (Fig. 1.2). *L. persoonii* was most virulent and had the largest mean lesion area; on average, it grew 3.9-fold faster than *B. obtusa* and 3.0-fold faster *P. amygdali*. There was no difference in disease susceptibility between cultivars for *P. amygdali*. However, there were significant differences between cultivars for both *B. obtusa* and *L. persoonii*. Based on the premise that lesion area is correlated with host resistance, ‘O’Henry’ appeared more susceptible to *B. obtusa* compared to the other cultivars, but

there were no significant susceptibility differences between the other cultivars. ‘O’Henry was least susceptible to *L. persoonii* when compared to the other cultivars. ‘Coronet’ was most susceptible to *L. persoonii* followed by ‘Contender.’

Discussion

Our survey determined that there are multiple fungal pathogens causing twig blight in the state of South Carolina. To our knowledge, this is the first survey of its kind conducted in the main peach production regions in South Carolina, although the pathogens were either known or suspected to exist in the region. Peach canker or constriction canker causing twig blight, was first described as a disease caused by *Fusicoccum amygdali* in 1941 (Haenseler and Daines 1941). Later, *F. amygdali* was reclassified as *P. amygdali* and was recognized as the species causing canker on peach in the southeastern U.S., including South Carolina (Farr et al. 1999). Initially, gummosis of peach was observed in Georgia, and the causal agent was described as *Botryosphaeria dothidea* (Moug. Ex Fr.) Ces. & De Not. (Weaver 1974). Later, *B. obtusa* was described in Georgia as another causal agent of the disease (Britton and Hendrix 1982). Generally, Leucostoma canker is considered a disease that occurs in cooler, more northern climates. However, multiple studies have included *L. persoonii* isolates collected from North Carolina and Florida (Adams et al. 2002; Hammar 1989; Alfieri et al. 1974) indicating pathogen presence in the southeastern United States. Available research indicates *P. amygdali* and *B.obtusa/B. dothidea* have been the most prevalent twig blight pathogens in the southeastern U.S. In fact, in the 2018 Southeastern Peach, Nectarine, and Plum Pest

Management and Culture Guide, *L. persoonii* was not even mentioned as an issue for peach producers, much less were there control options provided (Blaauw et al. 2018). Interestingly, our survey determined that *L. persoonii* was the most prevalent twig blight pathogen. This is not only a first formal report of this pathogen on peach in South Carolina, but also indicates that a shift may have taken place where *L. persoonii* appears now to be the most prevalent agent causing twig dieback. The superior virulence observed in our twig study suggests that *L. persoonii* has outcompeted other pathogens in the area. To our knowledge, there are no other published studies comparing the virulence of *L. persoonii* to other pathogens. Leucostoma canker and bacterial canker, caused by *Pseudomonas syringae*, have frequently been found together in peach (Alfieri et al. 1974). However, scaffold limb dieback in South Carolina has typically been attributed to bacterial canker as research has suggested that Cytospora canker is a secondary disease to bacterial canker (Ritchie and Clayton 1981). Because of these findings indicating the prevalence of *L. persoonii*, it would be worth revisiting the subject of bacterial canker being the main causal agent of scaffold limb dieback.

Several fungicidal active ingredients were tested to determine sensitivity of the pathogens *in vitro*. We found insensitivity to FRAC 7 fungicides in all of the pathogens. Recent research on *B. dothidea* also suggests the SDHI fungicides are ineffective in controlling mycelial growth *in vitro* (Dai et al. 2017). Because all pathogens were equally insensitive to the FRAC 7 fungicides, we suspect a natural, inherent insensitivity of these twig blight pathogens to this MOA. We do not suspect tolerance through fungicide selection. Other results indicate that some FRAC codes may be useful to protect trees

from infection from all wood pathogens identified in this study perhaps after hail, pruning, or during leaf drop when injuries to the tree increase infection risk. A previous study found that carbendazim (FRAC 1), prochloraz (FRAC 3), and difenoconazole (FRAC 3) were effective in preventing infection of *P. amygdali* in the field (Ji et al. 2013). Similarly, our results indicate that difenoconazole was effective in controlling *P. amygdali in vitro*. Another study indicated that captan (FRAC M04), chlorothalonil (FRAC M05), and azoxystrobin (FRAC 11) were most effective in reducing canker incidence and severity in the field but that no fungicide was able to achieve more than 75% control of *P. amygdali* (Lalancette and Robison 2002). Our assay did not include multisite fungicides such as captan or chlorothalonil but did achieve excellent control with FRAC 11 fungicides azoxystrobin and pyraclostrobin. Very little is known about the efficacy of modern fungicides for *L. persoonii* control. Previous research conducted on *L. persoonii* has shown that benomyl (FRAC 1) and captafol (FRAC M04) reduced disease incidence when applied in fall and the beginning of spring (Northover 1992). Another study on another Leucostoma canker pathogen, *L. cinctum*, conflicted with this and concluded that captafol was ineffective (Grosclaude 1985). However, now, many peach pathogens are resistant to benomyl (Bernstein et al. 1995; Penrose and Koffman 1977), and captafol is banned for use on peach. Captafol was also found to reduce incidence of fungal gummosis (Beckman et al. 2003). In that same study, the authors found that captan was less effective than captafol. In another study, propiconazole was most effective, and azoxystrobin had very low efficacy against *B. obtusa* (Ji et al. 2012). Our results agree that propiconazole achieved some control of *B. obtusa*, but the most control was achieved

with azoxystrobin. Conflicting results between studies and the lack of research comparing fungicides that are currently used in peach production make it difficult to definitively suggest fungicides to use for preventative control of twig blight pathogens. However, our research and previous studies provide evidence for the effectiveness of FRAC 1 and FRAC 11 fungicides in providing control for some of these twig blight fungi. Additionally, the FRAC M04 fungicides may also provide control for these pathogens based on previous research. Because this fungicide efficacy study was an *in vitro* experiment using artificial media, it is difficult to judge how these fungicides will behave in a field setting. In the future, these fungicides should be applied in a field situation to determine the field efficacy of these compounds.

Our results indicate that certain cultivars possessed differing levels of susceptibility to the different pathogens. However, the susceptibility was not consistent for cultivars across all pathogens. Several researchers have noted that many plant tissues form a lignosuberized layer upon wounding and its importance for fungal infection (Biggs and Miles 1985; Biggs and Britton 1988; Biggs and Miles 1988; Biggs and Peterson 1990; Rittinger et al. 1987). Previous research on cultivar susceptibility in *Leucostoma* spp. observed the potential influence of suberin formation in wound sites and cultivar susceptibility; essentially, cultivars that produced suberin sooner after damage to the wood were generally more tolerant to *Leucostoma* spp. infection (Biggs and Miles 1985, 1988; Biggs and Peterson 1990). One may hypothesize that this trend may extend to multiple fungal twig blight pathogens. Interestingly, however, our results indicate that not all cultivars display the same tolerance to *L. persoonii*, *P. amygdali*, and *B. obtusa*

(i.e. ‘O’Henry’ was least tolerant to *B. obtusa* and most tolerant to *L. persoonii*). Therefore, tolerance of peach cultivars to twig blight pathogens may not be based on the swift formation of a lignosuberized layer in the wooden tissue.

We selected the cultivars in this study based upon their susceptibility and tolerance to brown rot and bacterial spot. Brown rot resistance is associated with several genomic regions (Martínez-García et al. 2013; Pacheco et al. 2014). Bacterial spot resistance is believed to be associate with 14 quantitative trait loci (Yang et al. 2013). ‘Contender’ and ‘Coronet’ (tolerant and susceptible to brown rot, respectively) were the two least tolerant cultivars to *L. persoonii.* They were two of the most tolerant cultivars to *B. obtusa*. There was no difference between all cultivars for *P. amygdali*. These differences suggest that the same genetic basis for disease tolerance to brown rot in these cultivars is not the same mechanism governing disease tolerance to twig blight pathogens. ‘Summerprince’ and ‘O’Henry’ (tolerant and susceptible to bacterial spot, respectively) were the most tolerant to *L. persoonii*. ‘O’Henry’ was least tolerant and ‘Summerprince’ was one of the most tolerant to *B. obtusa*. In this case, *B. obtusa* had a similar tolerance profile to that of bacterial spot. However, *L. persoonii* does not. These differences also suggest that disease tolerance to *L. persoonii* is independent from that of bacterial spot in these cultivars but may be related to *B. obtusa*. However, in order to allow for maximum comparison between both cultivars and pathogens, the lesion areas in this study were measured at the same time for all twigs. For this reason, we believe that if *P. amygdali* and *B. obtusa* were allowed to grow for a longer period of time, we may have seen greater differences between cultivar susceptibility within species. In the future,

a follow-up study should be conducted that allows for longer growth of these pathogens to differentiate further between cultivars within pathogen. Our results provide promising information about the scope of twig blight pathogens in South Carolina as well as how we can develop tailored management strategies. In the follow-up studies for field testing of fungicides, it would be beneficial to include multi-site fungicides to provide more context to the chemical control of twig blight fungi. Similarly, while our detached branch inoculations provided promising information, field testing should be conducted to determine susceptibility of cultivars in a real-world setting. In summary, our study indicates that twig blight of peach in South Carolina and perhaps in other southeastern states, is caused by multiple pathogens but primarily by *L. persoonii*. This pathogen is capable of causing severe cankers on scaffold limbs leading to premature peach tree decline and thus developing management practices will be important.

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Table 1.1 Number of fungal pathogens collected from symptomatic twigs in South Carolina peach orchards.

Pathogen	Location						
	Chesnee	**Greer**	**McBee**	**Mountain Rest**	**Ridge Spring**	**York**	**Total**
Phomopsis amygdali	20	0	0	0	17	0	37
Botryosphaeria obtusa	0	2	2	1	0	6	11
***Cytospora* sp.**	4	0	0	0	0	2	6
Leucostoma persoonii	6	9	12	16	0	14	57
Total	30	11	14	17	17	22	111

Table 1.2 Sensitivity of pathogens to fungicides from four FRAC mode-of-action codes.

Pathogen	Isolate	EC_{50} values (µg/ml)						
		Thiophanate -methyl (FRAC 1)	**Difenoconazole (FRAC 3)**	**Propiconazole (FRAC 3)**	**Boscalid (FRAC 7)**	**Fluopyram (FRAC 7)**	**Pyraclostrobin (FRAC 11)**	**Azoxystrobin (FRAC 11)**
Phomopsis amygdali	C-1-16	0.13	0.289	1.189	>100	>100	<0.01	<0.01
	RS-5-16	0.156	0.093	1.182	>100	>100	<0.01	<0.01
	RS-10-16	0.13	0.049	1.031	>100	>100	<0.01	<0.01
Botryosphaeria obtusa	MB-5-16	0.064	0.026	0.42	>100	>100	<0.01	<0.01
	Y-6-16A	0.003	0.053	0.094	>100	>100	0.611	<0.01
	MR-14-16	0.002	0.207	0.585	56.819	>100	0.01	<0.01
Leucostoma persoonii	Y-2-16	0.128	0.858	0.929	>100	>100	0.158	<0.01
	MB-8-16	0.175	0.812	1.933	>100	>100	0.068	<0.01
	Y-17-16B	0.159	1.037	2.284	>100	>100	0.078	0.206

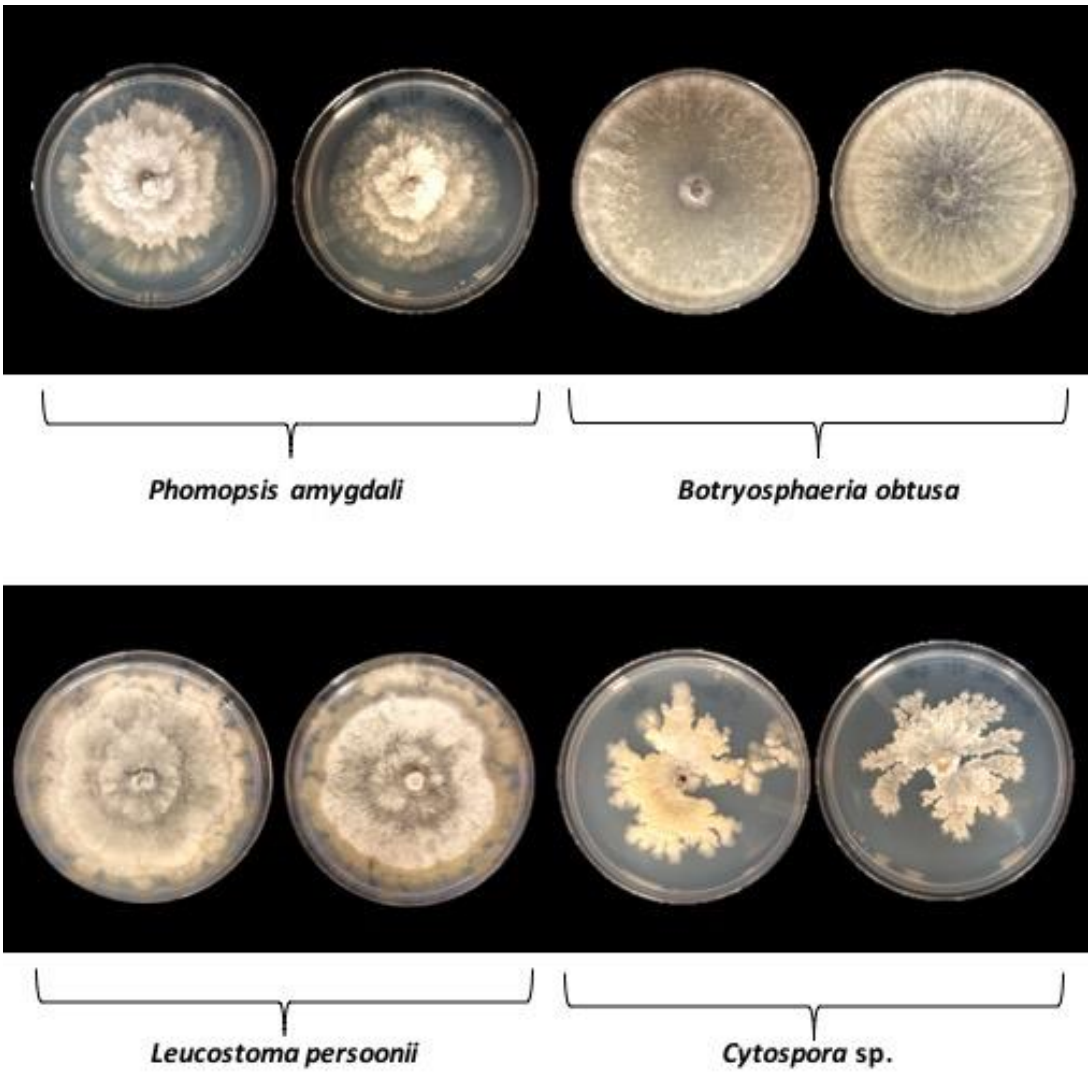

Figure 1.1 Representative morphologies of 2016 South Carolina twig blight isolates grown on PDA in the dark for 7 days at 22°C.

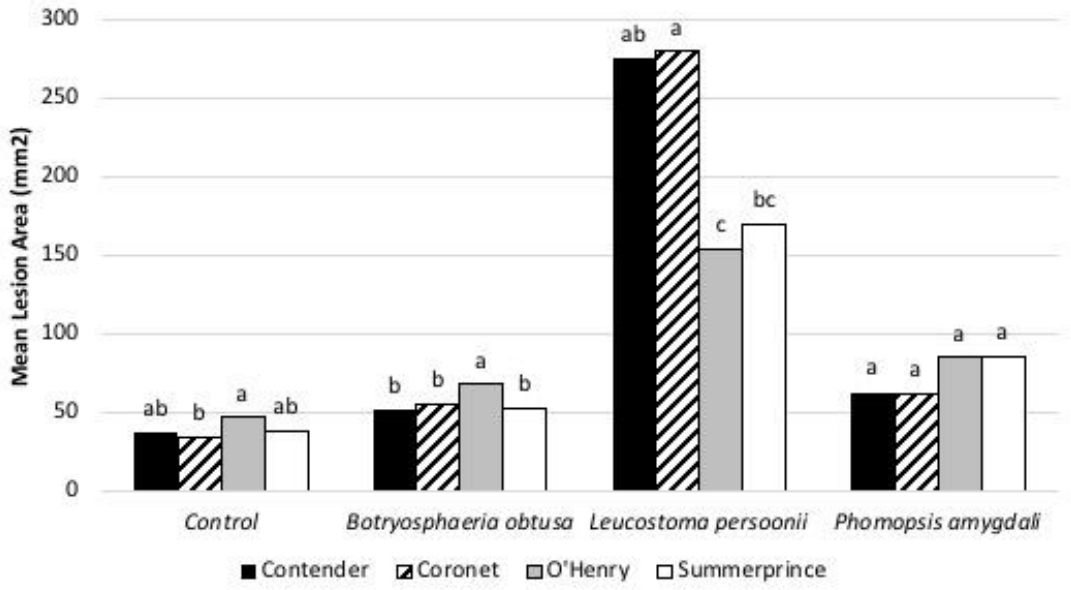

Figure 1.2 Mean lesion area caused by three pathogens in wood segments of four cultivars after 7 days of incubation at 22°C. Least square means were calculated based on a Tukey HSD post-hoc comparison ($\alpha = 0.05$). Different letters indicate significantly different responses within pathogen.

CHAPTER 3

GENETIC DIVERSITY AND SENSITIVITY TO FUNGICIDES IN *LEUCOSTOMA PERSOONII* OF PEACH

Abstract

Leucostoma persoonii (Nitschke) Höhn has been associated with recent premature peach tree decline in South Carolina, but very little is known about the pathogen or chemical control options. *L. persoonii* isolates were collected in 2016 and 2017 from symptomatic scaffold limbs and one-year-old peach wood from orchards in five locations in South Carolina. Six unique genotypes were identified based on substantial ITS1-5.8S-ITS2 sequence variability and classified G1 to G6. Three of the genotypes (G2, G3, and G6) were isolated in high frequency in both years. In addition to the genotypic variation, multiple phenotypes were observed between and within genotype. All genotypes were sensitive to thiophanate-methyl (FRAC 1) but exhibited slightly reduced sensitivity to propiconazole and difenoconazole (both FRAC 3). Boscalid and fluopyram (both FRAC 7) were ineffective at inhibiting mycelial growth of *L. persoonii* genotypes. Isolates were less sensitive to propiconazole and difenoconazole (FRAC 3) compared to thiophanate-methyl (FRAC 1). The data aquired in this study have implications for Leucostoma canker management in orchards.

Introduction

Leucostoma persoonii (Nitschke) Höhn [synonym: *Cytospora leucostoma* (Pers.) Sacc.] *Leucostoma cincta* (Fr. : Fr.) Höhn. (synonym: *Cytospora cincta* Sacc.) are important pathogens causing twig blight and cankers on peach trees in the United States (Adams et al. 2002; Hammar

1989; Willison 1933). Commonly referred to as Leucostoma canker, Cytospora canker, or perennial canker, the disease can reduce the fruit-bearing wood available on peach trees which, ultimately, can reduce the yield produced from a tree or an orchard. *L. persoonii* and *L. cincta* must infect through some opening in the wooden tissue such as a pruning wound, winter damage, or leaf scars around nodes (Luepschen et al. 1979; Tekauz and Patrick 1974). Initial symptoms of disease include brown and sunken canker formation around the infection area which then turns dark and dry with age (Willison 1933). Dieback occurs once the disease is advanced and causes the blockage and interruption of vascular tissues (Tekauz and Patrick 1974). The infected area later produces pycnidia that sporulate orange or yellow cirri which provide the inoculum that may infect additional branches and neighboring tree infection (Hildebrand 1947). Currently, cultural management strategies are suggested for management of Leucostoma canker. Research of current chemical products as a control method is lacking.

For years, these pathogens have been viewed as an opportunistic pathogen (Hildebrand 1947; Willison 1936). Additionally, they have also been associated with bacterial canker (*Pseudomonas syringae*) with pathologists suggesting that bacterial canker is the primary cause of dieback (Ritchie and Clayton 1981). Whether the pathogens are primary or opportunistic, they can rapidly spread through the wooden tissue of peach trees which causes yield loss for growers. Previous studies have also suggested that the disease is mainly an issue in the northern and western United States, but recent research has shown widespread occurrence in South Carolina peach orchards (Froelich and Schnabel 2018); *L. persoonii* was isolated in the highest frequency of all twig blight pathogens recovered. Previous research has also suggested the high genetic diversity of *Leucostoma*, particular within *L. persoonii* (Adams et al. 2002). This could have significant impacts on disease management because growers are combating a highly diverse

pathogen. The objectives of this study were to (i) evaluate the genetic diversity of *Leucostoma persoonii* isolates collected from South Carolina and (ii) assess their sensitivities to the fungicides most commonly used in peach production.

Materials and Methods

Sample collection and pathogen isolation. *Leucostoma persoonii* isolates were collected in 2016 from six locations in South Carolina, including Chesnee, Greer, McBee, Mountain Rest, Ridge Spring, and York. Necrotic tissue from underneath the periderm of one-year-old peach wood exhibiting pycnidia was excised and sterilized with 10% sodium hypochlorite for 1 minute and washed in sterilized distilled water for 1 minute in order to isolate wood fungi. Wood discs were plated in potato dextrose agar (PDA; 35.1 g Difco™ PDA, 900 ml H_2O) and incubated at 22°C in the dark until mycelia developed, and single-hyphal isolates were created and stored on filter paper as described previously (Hu, Cox, et al. 2011). A separate collection of *L. persoonii* was acquired in South Carolina in 2017 from Chesnee, Greer, McBee, Ridge Spring, and York. But in contrast to using one-year-old twigs as was done in 2016, isolates from scaffold limbs exhibiting severe dieback (greater than 80% but less than 100% of the limb was dead) were collected. Bark samples 10 x 10 cm in size were excised from the scaffold limbs to include the margin of necrotic and asymptomatic cambial tissue. The bark underwent similar processing as described above, and single-hyphal isolates were created and stored on filter paper.

DNA Extraction, PCR amplification, and sequencing of the Internal Transcribed Spacer regions 1 and 2. DNA was extracted according to a previous protocol (Chi et al. 2009) but mycelia were grown over cellophane to make removal easier. Polymerase chain reaction was

conducted to amplify the internal transcribed spacer (ITS) region 1, ribosomal 5.8S subunit, and ITS region 2 using primers ITS1-F and ITS4. PCR products underwent Sanger sequencing at the Arizona State University DNA Lab. These sequences were searched with the Basic Local Alignment Search Tool (nBLAST) from National Center for Biotechnology Information (NCBI) to assign fungal species identities, and Genious (version 11.0.4, Biomatters) was used for sequence assembly, alignment, and construction of phylogenetic trees. Reference isolates included in the phylogenetic tree were obtained from published research on *L. persoonii* and represent worldwide genetic variation of the species (Adams et al. 2002; Arhipova et al. 2011; Fotouhifar et al. 2010; Kepley et al. 2000; Singh et al. 2007).

Fungicide sensitivity assay. The half maximal effective concentration (EC_{50}) was used to determine sensitivities of *L. persoonii* isolates to fungicides from four Fungicide Resistance Action Committee (FRAC) mode-of-action (MOA) codes commonly used by growers in South Carolina peach orchards. Thiophanate-methyl (methyl benzimidazole carbamates [MBC], FRAC 1), propiconazole and difenoconazole (demethylation inhibitors [DMI], FRAC 3), boscalid and fluopyram (succinate dehydrogenase inhibitors [SDHI], FRAC 7), and pyraclostrobin and azoxystrobin (quinone outside inhibitors [QoI], FRAC 11) were used in the assay. These fungicides were selected because they were registered for disease management of peach in the United States at the time of this study. Malt extract agar (MEA; 10 g malt, 15 g agar, 1 L H_2O) was amended with the fungicides from FRAC 1, 3, and 11. For the FRAC 7 fungicides, minimal media (MM; 10 g glucose, 1.5 g K_2HPO_4, 2 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 2 g yeast extract, 12.5 g agar, 1 L H_2O) was used (Hu, Luo, et al. 2011). Salicylhydoxamic acid (SHAM) was also added to media for FRAC 11 to inhibit the alternative oxidase pathway. An initial study examined the sensitivity of five of the six *L. persoonii* genotypes from the 2016

collection to 0.1, 1, and 10 µg/ml active ingredient (ai). Three 4 mm mycelial plugs were extracted from the edges of one-week-old cultures and placed on each fungicide-amended and unamended control plate and incubated at 22°C in the dark until the three colonies were almost touching. At this point, the colonies were measured, and the average diameters were recorded. Subsequently, a final assay was conducted using refined concentrations based on the assessment of the preliminary measurements. The concentrations 0.1, 0.3, 1.0, 3, and 10 µg/ml ai were used for testing the thiophanate-methyl (FRAC 1); 0.1, 0.3, 1, 3, 10, 30 µg/ml ai for difenoconazole and propiconazole (FRAC 3); 3, 10, 30, and 100 µg/ml ai for boscalid and fluopyram (FRAC 7); and 0.01, 0.03, 0.1, 0.3, 1.0, and 3.0, 10, and 30 µg/ml ai for pyraclostrobin and azoxystrobin (FRAC 11). The assay was repeated with the refined concentrations. The average diameters recorded from the assay were used to calculate the EC_{50}.

Statistical analysis. JMP (SAS Institute, Inc., Cary, NC, USA) was used for all statistical modeling. A full-factorial ANOVA was conducted to determine the significance of fungicide, genotype, isolate, and their interactions to EC50. Fisher's LSD test was used to compare EC50 values between statistically significant ($\alpha = 0.05$) factors.

Results

A total of 57 *L. persoonii* isolates were collected from South Carolina peach twigs in 2016 from 5 of the 6 locations sampled (Table 2.1). The pathogen was isolated in the highest frequency from Mountain Rest (16 isolates), and no isolates were obtained from Ridge Spring. In 2017, 36 isolates were collected from scaffold limbs of 4 of the 5 locations sampled (Table 2.2). The pathogen was isolated in the highest frequency from Ridge Spring (11 isolates), and no isolates were obtained from York. Ribosomal DNA sequence analysis revealed the isolates to be

highest in sequence identity to *L. persoonii* from various countries. A phylogenetic tree of isolates selected from both years at random from each location showed high genetic diversity of *L. persoonii* isolates in the ITS1-5.8S-ITS2 region (Fig. 2.1). Representative isolates for the phylogenetic tree presented in Fig. 2.1 are a subset of isolates selected from another, larger phylogenetic tree that included all of the sequences from the 2016 and 2017 collections. The tree presented in this paper was created to give an accurate portrayal of genetic variation in a less complicated manner. Isolates were chosen for Fig. 2.1 from both years to represent all locations. First, two large clusters were identified (Cluster 1 and Cluster 2). A total of six groups (three from each cluster) were identified and designated as genotypes (G) 1 through 6. The isolates collected from twigs and branches in 2016 and 2017 clustered together in 5 of the 6 clusters (G1, G2, G3, G4, and G6) (Fig. 1.2). Surprisingly, the isolates from other parts of the country and international isolates clustered together with our isolates indicating the genetic diversity observed in our national and international collection was represented in our local, South Carolina collection. Representative isolate growth for each genotype indicate that variation of morphology is present both between and within genotype (Fig. 2.2).

Differences in sensitivity of combined *L. persoonii* isolates to various fungicides were observed ($P = 0.002$), but the genotype and the interaction between genotype and fungicide were not statistically significant. Across all genotypes, the half maximal effective concentration (EC_{50}) values for thiophanate-methyl (FRAC 1) ranged from 0.021 to 0.513 µg/ml ai (Table 2.3). EC_{50} values within genotypes for this fungicide was generally consistent with some variation occurring among isolates of G3. The EC_{50} values for difenoconazole (FRAC 3) ranged from 0.120 to 2.912 µg/ml ai and for propiconazole it ranged from 0.394 to 2.879 µg/ml. EC_{50} values for thiophanate methyl were significantly lower compared to those for both DMI fungicides, but

no significant difference was found between EC_{50} values for difenoconazole and propiconazole. For boscalid and fluopyram the EC_{50} values were greater than 100 µg/ml ai, indicating resistance of the isolates tested to this class of fungicides. Similarly, the assay was inadequate to accurately represent the dose-response to the FRAC 11 fungicides. Therefore, these data were excluded from Table 2.3. Additionally, statistical programming was only run on fungicides that inhibited fungal growth of *L. persoonii* within the assay.

Discussion

Our survey of South Carolina peach orchards in 2016 and 2017 revealed the presence of several highly diverse genotypes within *L. persoonii*. Similarly, previous research conducted on the *Leucostoma* genus revealed high genetic diversity within *L. persoonii* ITS sequences, indicating that *Leucostoma* isolates that genetically differ substantially from each other are still considered the same species (Adams et al. 2002). The ITS region has been considered the universal DNA barcode for the kingdom Fungi (Schoch et al. 2012) unless other genes have been identified as a better identifier to the species level of a fungal species (Geiser et al. 2004; Staats et al. 2005). Intraspecific variation can vary with some species having little to no variation and others having extremely high variation (Nilsson et al. 2008). It was suggested that the canonical 3% threshold (meaning that variation higher than this implies an isolate is another species) is too rigid and that a more specific, personal knowledge of a taxonomic group is required when analyzing ITS sequence information. In the case of *L. persoonii*, the understanding is that an inherent intraspecific variation exists (Adams et al. 2002); however, to what extent is unknown. A multi-gene analysis combined with morphological comparisons is preferred when examining *L. persoonii* isolates of varying genetic background.

Genotypes G2, G3, and G6 were isolated in high frequency in both years, indicating that these may be more virulent compared to other, less-frequently isolated genotypes. We also observed substantial morphological differences both between and within genotypes. This indicates that genetic diversity is likely ubiquitous within the genome between and among the genotypes identified in this study. Although interesting, the phenotypic and genotypic variations within and between genotypes will create a challenge when attempting to identify the pathogen to the species or genotype level. Reference isolates from around the world clustered within the diversity found in South Carolina isolates, a phenomenon that cannot be easily explained. Peach planting stock movement only occurs within the United States and never between the United States and other countries due to strict regulations.

We identified differences in fungicide efficacy between active ingredients for *L. persoonii* as a whole but found no significant effect of the genotype. We attributed boscalid and fluopyram insensitivity to an intrinsic trait of the species and not a result of fungicide selection because of the overwhelming insensitivity to both active ingredients across all genotypes and the lack of sensitive baseline isolates. Additionally, we determined our assay was not adequate to test azoxystrobin and pyraclostrobin (both FRAC 11). Thiophanate-methyl was the most effective inhibitor of *L. persoonii* mycelial growth, followed by difenoconazole and propiconazole. Most previous studies have focused on the sensitivity of *L. persoonii* to multi-site fungicides. One older study in particular found ferbam (FRAC M03) and sulfur (FRAC M02) to be effective in inhibiting spore germination *in vitro* (Dhanvantari 1968). Another found benomyl (FRAC 1) and captafol (FRAC M04) lessened Leucostoma canker disease incidence when it was applied both in fall and spring (Northover 1992). However, the efficacy of captafol was not always confirmed (Grosclaude 1985). While captafol can no longer be used on peach due to

changes in regulations and many peach pathogens are resistant to benomyl (Bernstein et al. 1995; Penrose and Koffman 1977), active ingredients with identical MOAs to benomyl are still available.

In conclusion, *L. persoonii* is a widespread, genetically diverse pathogen of twigs and scaffold limbs in South Carolina peach orchards. The information from this study provides growers with options to protect trees from infection in orchards that are affected. However, concrete recommendations have not been established.

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Table 2.1. Number of isolates for each Leucostoma persoonii genotype collected from symptomatic twigs in South Carolina peach orchards in 2016.

	Location						
***Leucostoma persoonii* Group**	**Chesnee**	**Greer**	**McBee**	**York**	**Ridge Spring**	**Mountain Rest**	**Total**
G1	0	1	2	1	0	0	4
G2	3	3	6	7	0	1	20
G3	1	0	3	6	0	1	11
G4	0	1	0	0	0	0	1
G5	0	0	1	0	0	0	1
G6	2	4	0	0	0	14	20
Total	6	9	12	14	0	16	57

Table 2.2. Number of isolates for each *Leucostoma persoonii* genotype collected from symptomatic scaffold limbs in South Carolina peach orchards in 2017.

	Location					
***Leucostoma persoonii* Group**	**Chesnee**	**Greer**	**McBee**	**York**	**Ridge Spring**	**Total**
G1	1	2	0	0	0	3
G2	2	1	4	0	4	11
G3	1	1	0	0	2	4
G4	0	0	0	0	2	2
G5	0	0	0	0	0	0
G6	4	7	0	0	5	16
Total	8	11	4	0	13	36

Table 2.3. Sensitivity of *Leucostoma persoonii* genotypes to fungicides from four FRAC codes.

Genotype	Isolate	EC_{50} values (µg/ml)				
		Thiophanate-methyl (FRAC 1)	**Difenoconazole (FRAC 3)**	**Propiconazole (FRAC 3)**	**Boscalid (FRAC 7)**	**Fluopyram (FRAC 7)**
G1	MB-3-16	0.352	0.853	0.598	>100	>100
	MB-15-16	0.513	0.596	0.817	>100	>100
	G-19-16	0.185	0.120	0.394	>100	>100
G2	C-26-16	0.416	1.122	1.716	>100	>100
G3	MB-2-16	0.096	1.779	2.116	>100	>100
	MB-8-16	0.021	1.666	2.967	>100	>100
	MR-13-16	0.467	1.311	1.123	>100	>100
	Y-17-16B	0.464	1.949	1.821	>100	>100
G5	MB-11-16	0.361	0.184	0.832	>100	>100
G6	C-27-16	0.317	0.752	1.551	>100	>100
	G-13-16	0.335	0.874	1.596	>100	>100
	MR-10-16	0.347	2.912	2.879	>100	>100
		b[x]	a	a		

[x]Letters indicate significant differences of combined EC_{50} values between fungicides that reached complete inhibition of mycelial growth ($\alpha = 0.05$).

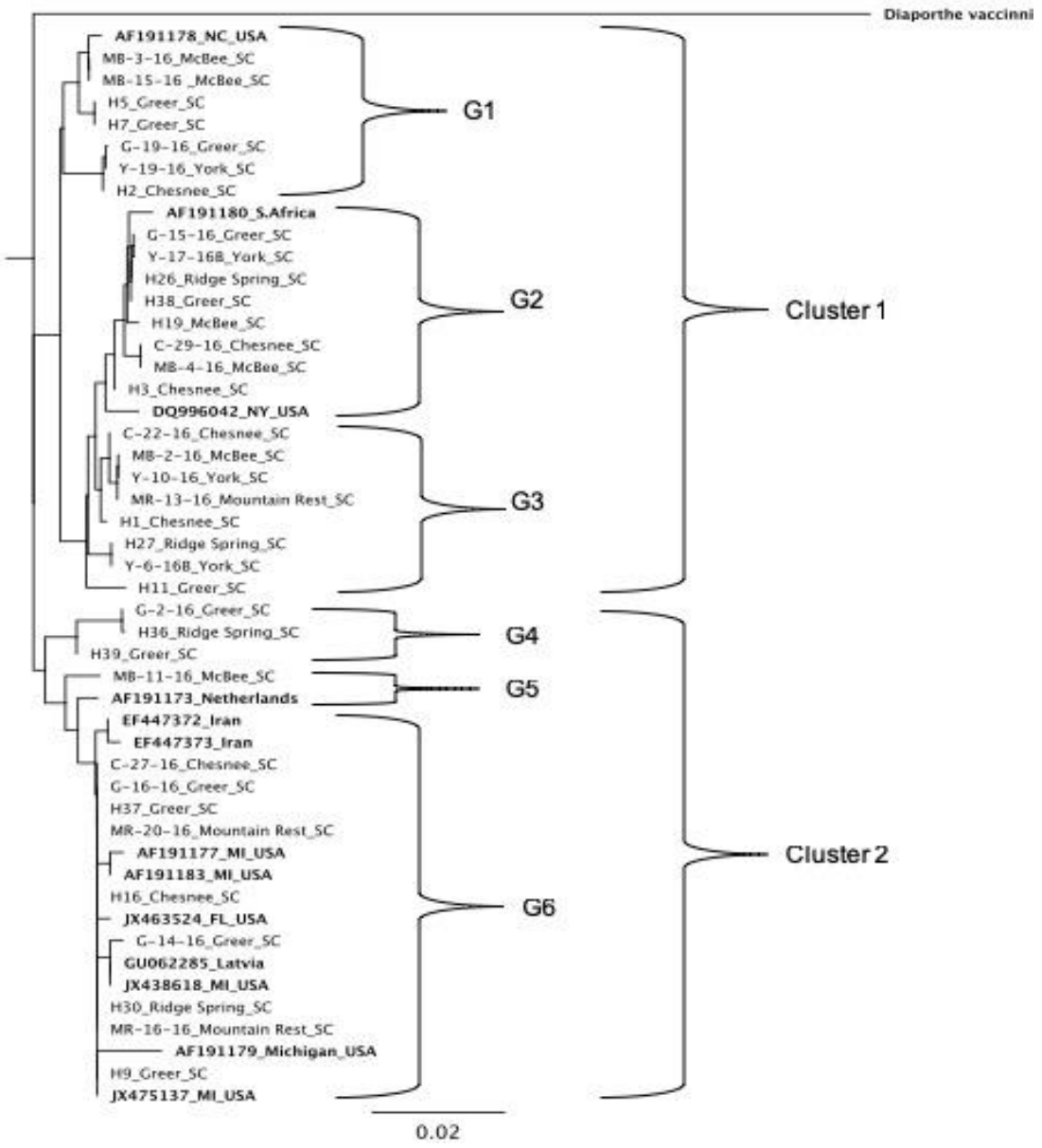


Figure 2.1. Neighbor-joining phylogenetic tree based on the ITS1-5.8S-ITS2 region of *Leucostoma persoonii* isolates collected from South Carolina in 2016 and 2017. Bolded isolates were obtained from the National Center for Biotechnology information Nucleotide BLAST (NCBI nBLAST) and were selected from published research to reflect the geographical locations of *L. persoonii* worldwide.

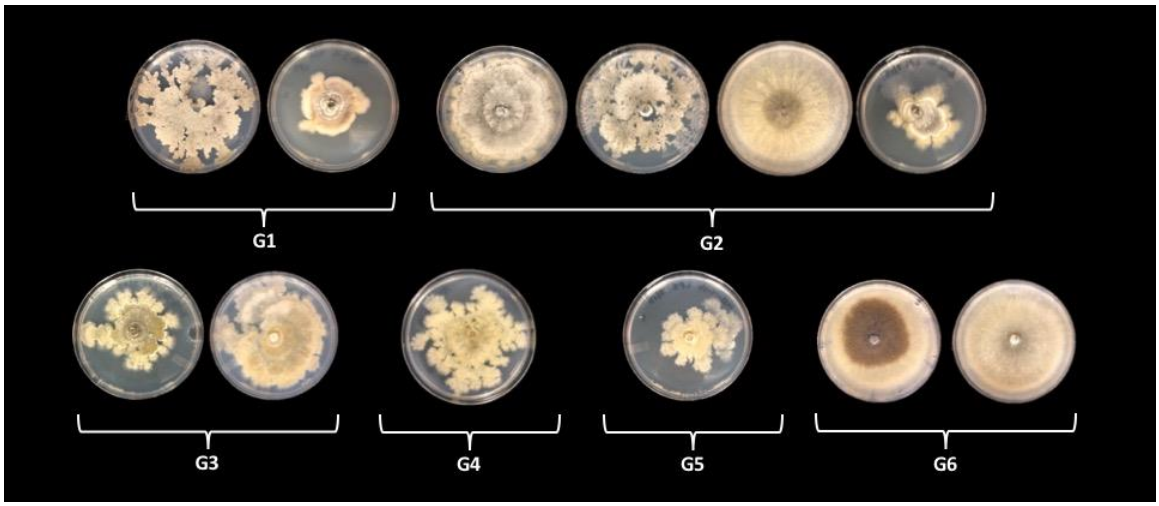
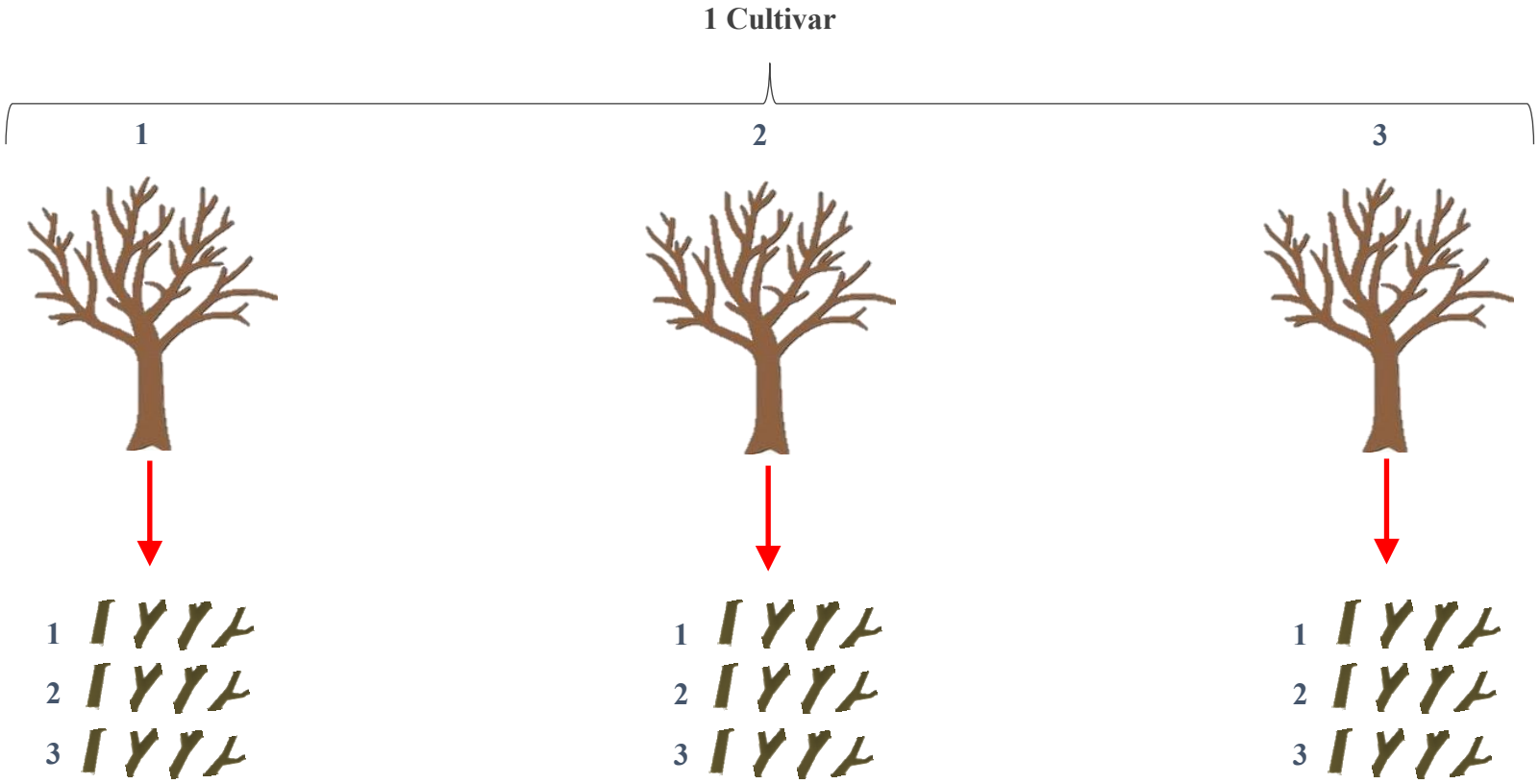


Figure 2.2. Representative morphologies of 2016-2017 South Carolina *Leucostoma persoonii* genotypes (G) grown on PDA in the dark for 7 days at 22°C. Genotypes were distinguished based on sequence grouping in a phylogenetic tree.

APPENDICES

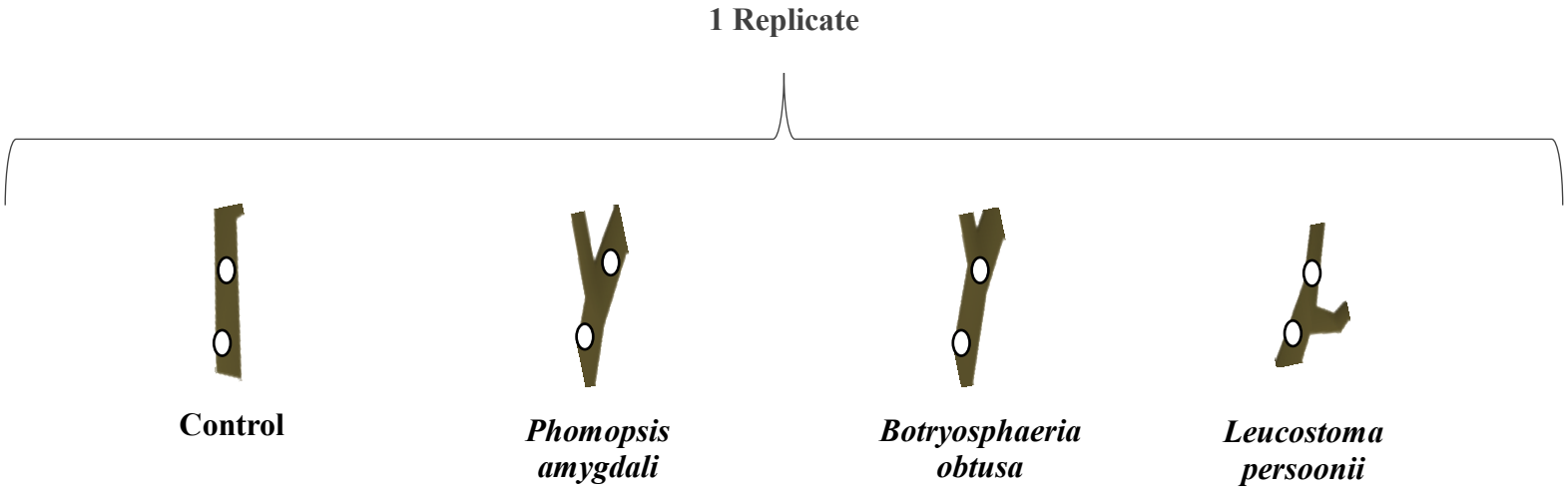
APPENDIX A

Experimental design for the cultivar susceptibility detached branch assay. Wood was collected from three trees from each cultivar, and there were three experimental replicates per tree.



APPENDIX B

Experimental design of the cultivar detached branch assay. Within one replicated, there were four treatments, and there were two inoculations of the same treatment per twig segment.



APPENDIX C

Fungicide sensitivity profiles of various fungicides represented by green (sensitive), yellow, (moderately sensitive), and red (tolerant).

Pathogen	FRAC 1	FRAC 3		FRAC 7		FRAC 11	
	Thiophanate-methyl	Difenoconazole	Propiconazole	Boscalid	Fluopyram	Pyraclostrobin	Azoxystrobin
Phomopsis amygdali							
Botryosphaeria obtusa							
Leucostoma persoonii							

Sensitive Moderately Sensitive Tolerant